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Phase Variation of Hemoglobin Utilization in *Neisseria gonorrhoeae*

CHING-JU CHEN,[1] CHRISTOPHER ELKINS,[1,2] AND P. FREDERICK SPARLING[1,2]*

Departments of Medicine[1] and Microbiology and Immunology,[2] School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599

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Most *Neisseria gonorrhoeae* isolates are unable to use human hemoglobin as the sole source of iron for growth (Hgb^-), but a minor population is able to do so (Hgb^+). This minor population grows luxuriously on hemoglobin, expresses two outer membrane proteins of 42 kDa (HpuA) and 89 kDa (HpuB), and binds hemoglobin under iron-stressed conditions. In addition to the previously reported HpuB, we identified and characterized HpuA, which is encoded by the gene *hpuA*, located immediately upstream of *hpuB*. Expression of both proteins was found to be controlled at the translational level by frameshift mutations in a run of guanine residues within the *hpuA* sequence encoding the mature HpuA protein. The "on-phase" hemoglobin-utilizing variants contained 10 G's, while the "off-phase" variants contained 9 G's. Insertional *hpuB* mutants of FA19 Hgb^+ and FA1090 Hgb^+ no longer expressed HpuB but still produced HpuA. A polar insertional mutation of the upstream *hpuA* gene in FA1090 Hgb^+ eliminated production of both HpuA and HpuB, whereas a nonpolar insertional mutant expressed HpuB only. Insertional mutagenesis of either *hpuA* or *hpuB* or both substantially decreased the hemoglobin binding ability of the FA1090 Hgb^+ variant and prevented growth on hemoglobin plates. Therefore, both HpuA and HpuB were required for the utilization of hemoglobin for growth.

Mammalian hosts use iron-binding proteins and iron-sequestering compounds to maintain free iron at a level that is too low for the growth of invading bacterial pathogens (52). Several pathogenic organisms have developed specific mechanisms for iron acquisition which are induced under conditions of iron limitation. The genital mucosal pathogen *Neisseria gonorrhoeae* is known to use iron from lactoferrin and transferrin for growth (37, 38). The ability to use lactoferrin and transferrin is due to specific receptors (reviewed in reference 11). Gonococci also are able to utilize heme and certain heme-containing proteins as iron sources (15, 38). Two heme-binding proteins (99 and 44 kDa) have been isolated from the total membranes of gonococcal clinical isolates grown under iron-limited conditions (26, 29). Nevertheless, the mechanism of iron uptake from heme-containing proteins is unclear.

Mickelsen and Sparling (38) showed that 18 of 29 gonococcal strains studied were capable of using hemoglobin for growth. Lee and Hill (28) detected hemoglobin binding activity from *Neisseria meningitidis* grown under iron-limited conditions. More recently, two different meningococcal hemoglobin receptors have been described at the molecular level. Stojiljkovic et al. (49, 50) cloned a gene encoding an iron-regulated outer membrane protein, HmbR, which has amino acid similarity with the family of TonB-dependent membrane receptors. Lewis and Dyer (30) and Lewis et al. (31) described *hpuAB*, the hemoglobin-haptoglobin utilization operon of *N. meningitidis*, and suggested that HpuA and HpuB constitute a two-component receptor analogous to the bipartite transferrin receptor TbpB-TbpA (formerly named Tbp2-Tbp1) (reviewed in reference 11). Predicted amino acid sequences of *N. meningitidis* HpuA and HpuB indicate that, like TbpB and TbpA, HpuB belongs to the TonB-dependent family of high-affinity transport proteins and HpuA is probably a lipoprotein (31).

* Corresponding author. Mailing address: Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7005. Phone: (919) 966-4468. Fax: (919) 966-5775. E-mail: zman@med.unc.edu.

We previously reported that all tested gonococcal strains can use hemoglobin as a sole source of iron for growth by switching from a non-hemoglobin-utilizing phenotype (Hgb^-) to a hemoglobin-utilizing phenotype (Hgb^+) in vitro. In order to distinguish these two phenotypes of the same strain, we classified gonococci able to grow on hemoglobin-Desferal plates as Hgb^+ variants and those unable to grow on hemoglobin-Desferal plates as Hgb^- variants (8). The ability to use hemoglobin for growth appears to be a phase-varying phenomenon, because Hgb^+ variants arise from Hgb^- parents at a frequency of about 1×10^{-4} to 2×10^{-3} (8). In this communication, we describe the mechanism underlying the phase variation of hemoglobin utilization and the characteristics of a 42-kDa HpuA protein encoded by the gonococcal *hpuA* gene. We also previously reported that insertional mutants that no longer expressed the 89-kDa HpuB protein cannot utilize hemoglobin to support growth but still grow on heme (8). Now we report on the requirement for HpuA in the utilization of hemoglobin for growth by *N. gonorrhoeae.*

MATERIALS AND METHODS

Bacterial strains and growth conditions. *N. gonorrhoeae* strains used are listed in Table 1. Both Hgb^+ and Hgb^- variants of FA1090 and FA19 were used. Growth conditions were the same as those described by Chen et al. (8), with the exception that the iron chelator Desferal (deferoxamine mesylate; CIBA Pharmaceutical Co.) was used at a 100 μM final concentration for FA1090 and insertional mutants of FA1090. The abilities of FA1090 Hgb^- and Hgb^+ variants and their *hpuA* and/or *hpuB* insertional mutants to grow on heme were tested on modified gonococcal medium base (GCB) agar plates (8) containing bovine hemin chloride (Sigma) at 5 mg/liter and Desferal at 100 μM. Antibiotic selection in gonococci employed erythromycin at 1 mg/liter for mini-Tn*3*erm insertions (30), spectinomycin at 100 mg/liter for the Ω interposon insertions (44), and kanamycin at 60 mg/liter for the *aphA*-3 cassette insertions (35).

Sequencing the gonococcal *hpuA* gene. Double-stranded PCR products, purified with a Geneclean II kit (Bio 101, Inc.), were sequenced directly. Chromosomal DNAs of FA1090 Hgb^+ and FA1090 Hgb^- were used as templates for PCRs. Initially, the putative open reading frame of FA1090 *hpuA* was identified from the database of the Gonococcal Genome Sequencing Project (Advanced Center for Genome Technology, University of Oklahoma) by searching for the coding sequence of the N terminus of FA1090 HpuB (8). The 19-mer upstream PCR primer (hpu.01, GCAGGCACGTCCGATTTCC) was designed based on the sequence 108 bp upstream of the ATG codon of the putative *hpuA* open

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Gonococcal strains		
FA1090	Wild type; Hgb$^+$ and Hgb$^-$ variants	41
FA19	Wild type; Hgb$^+$ and Hgb$^-$ variants	38
FA6929	FA1090 Hgb$^+$; *hpuB*::mTn*3*erm	8
FA6930	FA19 Hgb$^+$; *hpuB*::mTn*3*erm	8
FA6981	FA1090 Hgb$^-$; *hpuA*::Ω	This work
FA6982	FA1090 Hgb$^+$; *hpuA*::Ω	This work
FA6983	FA1090 Hgb$^+$; *hpuA*::*aphA*-3	This work
Plasmids		
pSM85k	850-bp *Apo*I fragment of *hpu* in pHS8	30
pSM85kE	pSM85k::mTn*3*erm; source for *hpuB*::mTn*3*erm	30
pHP45Ω	Source for Ω interposon (Sm^r)	44
pUC18K	850-bp *aphA*-3 cassette in pUC18 (Km^r)	35
pUNCH258	*hpuA* clone from FA1090 Hgb$^-$ in pCRII	This work
pUNCH259	*hpuA* clone from FA1090 Hgb$^+$ in pCRII	This work
pUNCH260	*Nar*I-*Xba*I segment from pUNCH 258; *hpuA* fragment with no poly(G) tract	This work
pUNCH268	*Ppu*MI-cut pUNCH260 with *Sma*I-cut Ω in pBluescript II KS$^+$	This work
pUNCH271	*Ppu*MI-cut pUNCH260 with *Sma*I cut-*aphA*-3 in pBluescript II KS$^+$	This work

reading frame (Fig. 1). A 20-mer downstream primer (hpu.14, ACAAAACCC AAAAACTCGGAC) was designed, based on the N-terminal amino acid sequence of HpuB, to generate PCR DNA fragments covering the entire *hpuA* gene (Fig. 1). Two PCRs were carried out for each variant. One used purified chromosomal DNA, and the other used boiled cells, as the template. PCR products were pooled for sequencing. Both strands of the PCR-generated *hpuA* DNA were sequenced by using hpu.01, hpu.14, and four additional internal primers.

In order to verify that hemoglobin-utilizing and non-hemoglobin-utilizing variants of *N. gonorrhoeae* contained different numbers of guanine (G) residues in the poly(G) tract of *hpuA*, guanine residues were enumerated from sequences of PCR products covering the poly(G) tract but not the entire *hpuA*. Two sets of templates were prepared from each variant of FA19 and FA1090. One was the purified chromosomal DNA, and the other was boiled cells. A 19-mer downstream primer (hpu.02, CATCTTCAAAACACCCGGC) was designed based on the sequence 247 bp downstream from the end of the poly(G) tract (314 bp from the initial ATG codon shown in Fig. 1). PCRs using the primer pair hpu.01 and

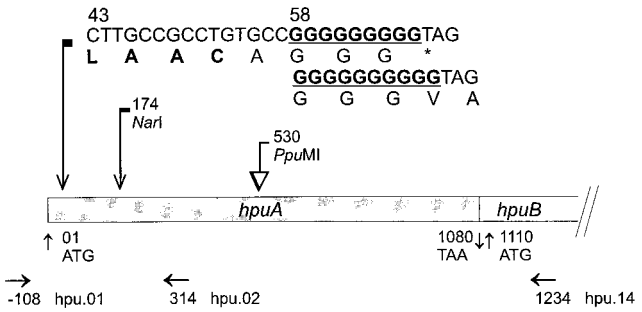

FIG. 1. Descriptive map of FA1090 *hpuA*. Numbers indicate positions of first nucleotides. The open reading frames (ORFs) for *hpuA* and *hpuB* start at nucleotide positions 01 and 1,110, respectively. Beginning from position 43 is a consensus coding sequence for a type-II signal peptidase cleavage site, LAAC. A run of multiple guanine residues was located in *hpuA*, starting from position 58. The asterisk indicates a stop codon. A 1,130-bp fragment was isolated between the *Nar*I site in *hpuA* and an *Xba*I site in the vector for mutagenesis. The *Ppu*MI site was the insertion site for making various mutants. The primer pair, hpu.01 and hpu.14, was used in PCRs for the cloning of *hpuA*. The other primer pair, hpu.01 and hpu.02, was used to generate PCR products for enumerating guanine residues in the poly(G) tract.

hpu.02 were expected to generate DNA fragments of 439 bp from Hgb$^-$ variants and of 440 bp from Hgb$^+$ variants. Sequencing of PCR-generated partial FA1090 *hpuA* fragments was carried out on both strands with primers hpu.01 and hpu.02. Sequencing of PCR-generated partial FA19 *hpuA* fragments was carried out on single strands only.

Oligonucleotides were synthesized at the Lineberger Comprehensive Cancer Center DNA Synthesis Facility of the University of North Carolina—Chapel Hill. DNA sequencing was carried out at the Automated DNA Sequencing Facility of the University of North Carolina—Chapel Hill with an Applied Biosystems model 373 DNA sequencer by use of the Tag DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems).

Cloning of the *hpuA* gene. Routine molecular cloning techniques as described by Sambrook et al. (47) were used. Plasmid DNAs were isolated by either the Wizard Minipreps DNA purification system (Promega) or the Qiagen DNA purification system. Chromosomal DNA from *N. gonorrhoeae* was purified by the method of Stern et al. (48). The 1,360-bp PCR DNA made from the FA1090 Hgb$^-$ template and the 1,361-bp PCR DNA made from the FA1090 Hgb$^+$ template were inserted into the pCRII vector by using the TA cloning kit (Invitrogen) to yield pUNCH258 and pUNCH259, respectively.

Insertional mutagenesis of the *hpuA* gene. (i) Construction of polar *hpuA* insertional mutants. In order to avoid potential variations in the poly(G) region during experimental manipulation of *hpuA*, insertional mutagenesis of *hpuA* was carried out within a *Nar*I-*Xba*I fragment isolated from pUNCH258 (Fig. 1). The *Nar*I site was located 108 bp downstream of the poly(G) tract, and the *Xba*I site was present in the polylinker of pCRII. The *Nar*I-*Xba*I fragment was subcloned into the pBluescript II KS$^+$ phagemid (Stratagene) digested with *Xba*I-*Acc*I to yield pUNCH260. In order to avoid blocking of *Ppu*MI digestion by Dcm methylase activity, pUNCH260 was moved from *Escherichia coli* DH5αMCR (Bethesda Research Laboratories) to GM2163 (New England Biolabs). The 2-kb Ω interposon fragment isolated from *Sma*I-cut pHP45Ω (44) was ligated with pUNCH260, digested with *Ppu*MI, and filled in with the Klenow fragment of DNA polymerase I (Fig. 1) to yield pUNCH268. pUNCH268, containing an Ω insert in the *hpuA* fragment, was then transformed into FA1090 Hgb$^-$ and FA1090 Hgb$^+$ to generate FA6981 and FA6982, respectively. Transformant colonies were screened for resistance to spectinomycin at 100 mg/liter and the inability to grow on hemoglobin-Desferal plates.

(ii) Construction of a nonpolar *hpuA* insertional mutant. A mutagenic cassette designed to be inserted into a gene of an operon without affecting transcription of downstream genes (35) was adopted for our construction of a nonpolar *hpuA* mutant. The 800-bp *aphA*-3 insert cassette was isolated from pUC18K by *Sma*I digestion (35) and was ligated to *Ppu*MI-digested pUNCH260 to generate pUNCH271. pUNCH271 was then transformed into FA1090 Hgb$^+$ to create FA6983. Transformant colonies were screened for resistance to kanamycin at 60 mg/liter and the inability to grow on hemoglobin-Desferal plates.

Enumeration of G residues. The cloned *hpuA* gene in pUNCH258 and pUNCH259 was sequenced with M13 reverse primer (Invitrogen). The guanine residues in *hpuA* from FA6981, FA6982, and FA6983 were enumerated from PCR products generated by the primer pair hpu.01 and hpu.02 in order to confirm that gonococcal transformants containing the mutagenized fragment of *hpuA* kept the same number of guanine residues in the poly(G) tract as was present in the parents. Primer hpu.01 was used in sequencing those partial *hpuA* fragments from mutants.

DNA hybridization. Southern blots were utilized to confirm that allelic exchange occurred as expected. Chromosomal DNAs of FA1090 Hgb$^-$, FA1090 Hgb$^+$, and *hpuA* or *hpuB* insertional mutants of FA1090 Hgb$^+$ (FA6982, FA6983, and FA6929) were digested with *Cla*I and separated on 0.7% agarose gels. Gels were transferred bidirectionally to Magnagraph nylon membranes (Micron Separations). Blots were probed, as described in Results, with random-primed digoxigenin-dUTP-labeled DNA and were developed according to the Genius labeling and detection instructions (Boehringer Mannheim Biochemicals).

Peptide synthesis and immunization. A 20-mer peptide was designed based on the predicted strong antigenicity of the last 17 amino acids of the C terminus of HpuA (CGG-EKKLDDTSQDTNHLTKQ). Another peptide (CGG-ADPAPQ SAQTLN) was designed previously based on the N-terminal amino acid sequence of HpuB (8). Peptide synthesis was performed at the University of North Carolina Program in Molecular Biology and Biotechnology MicroProtein Chemistry Facility with a multiple peptide synthesizer (Rainin Symphony). Peptide synthesis and immunization followed the procedures described by Elkins (16) and Chen et al. (8).

Affinity purification of antibodies raised against the N-terminal peptide of HpuB and the C-terminal peptide of HpuA. Postimmune antipeptide sera for HpuB (8) and HpuA were each affinity purified to eliminate reactivity to antigens other than HpuA and HpuB. A modification of a previously published method as reported by Elkins et al. (17) was used for the affinity purification of antipeptide antibodies on immobilized peptide coupled on thiopropyl-agarose columns (Sigma) (42).

Preparations of whole-cell dot blots and outer membranes from gonococci grown under iron-replete and iron-stressed conditions. Gonococci were grown in iron-sufficient and iron-limited media. Gonococcal medium broth containing Kellogg's supplement I was inoculated from overnight plate-grown gonococcal cultures (Hgb$^-$ on GCB plates and Hgb$^+$ on hemoglobin-Desferal plates). After

one mass doubling, cultures were split. Desferal was added to a 100 μM concentration to induce iron starvation in one flask, whereas supplement II (ferric nitrate at 12 μM) was added to the other flask. After 4 h of growth with either the chelator or the iron supplement, cells were harvested by centrifugation. The density of harvested cells was adjusted to an optical density of 0.2 at 600 nm (approximately 2×10^8 CFU/ml) with phosphate-buffered saline. Dot blots were prepared from wells loaded with 100 μl of the adjusted cell suspensions. Total membranes and outer membranes were prepared as previously described (16). The protein content was determined by a bicinchoninic acid assay (Pierce).

Purification of hemoglobin-binding proteins from total membranes. Purification of hemoglobin-binding proteins used bovine hemoglobin-agarose (chromatography specialty resin) supplied by Sigma. Purification with solid-phase hemoglobin-agarose followed a procedure described by Elkins (16). Hemoglobin-binding proteins were eluted in Laemmli sample buffer for analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting).

Whole-cell dot blot hemoglobin binding assay. FA1090 Hgb^+, FA6929, and *hpuA* insertional mutants of FA1090 Hgb^+ (FA6982 and FA6983) were examined for their ability to bind hemoglobin in a whole-cell dot blot assay. Human hemoglobin was biotinylated with *N*-hydroxysuccinimide–SS–Biotin (21331D; Pierce) according to the manufacturer's instructions at a molar ratio of 23:1 at 4°C for 2 h. The unbound biotin was removed by dialysis overnight in phosphate-buffered saline at 4°C. The binding was detected by a colorimetric procedure using alkaline phosphatase-conjugated streptavidin (Pierce) as the secondary reagent.

Western blot analysis of membrane proteins. SDS-PAGE and Western blotting of total membrane proteins, outer membrane proteins, and hemoglobin-binding proteins purified from total membranes were performed as described by Harlow and Lane (20). One nitrocellulose membrane each was prepared for Western blotting of total membrane, outer membrane, and hemoglobin-binding proteins. These membranes were cut into upper and lower panels along a line drawn between 55 and 60 kDa. The upper panels were probed with affinity-purified serum raised against the N-terminal peptide of HpuB (8), and the lower panels were probed with affinity-purified serum raised against the C-terminal peptide of HpuA.

RESULTS

Gonococcal *hpuA* gene. We obtained the sequence of *hpuA* from both FA1090 Hgb^- and FA1090 Hgb^+ by sequencing PCR products of the primer pair hpu.01 and hpu.14 directly. Data containing DNA sequence of FA1090 *hpuA* have been released incrementally by the *N. gonorrhoeae* Genome Sequencing Project. The sequence identical to our FA1090 Hgb^- *hpuA* sequence was released as part of the February 1997 update. Since the sequence of the FA1090 *hpuAB* operon is now readily available from the database of the project (the University of Okalahoma's Advanced Center for Genome Technology at http://www.dna1.chem.uoknor.edu) and from GenBank (accession no. AF031495), the *hpuA* sequence is not listed in detail in this communication.

FA1090 *hpuA* was located immediately upstream from *hpuB*, and there was no obvious promoter sequence in the 27 nucleotides that separate *hpuA* and *hpuB*. In the hemoglobin-utilizing variant, the *hpuA* gene had an open reading frame of 1,079 bp, 56 bp longer than that in the hemoglobin-haptoglobin-utilizing *N. meningitidis* strain DNM2 (31) (GenBank accession no. U73112). The putative Fur box in the promoter region of FA1090 *hpuA* was identical to that reported for DNM2. Forty-three base pairs downstream from the ATG codon was the beginning of a consensus sequence for a type-II signal peptidase cleavage site, LAAC (21) (Fig. 1). Processing of the gonococcal HpuA at the LAAC site would produce a mature peptide of 343 amino acids at a molecular size of 36 kDa. The isoelectric point of unmodified mature peptide was predicted to be 6.77. The putative HpuA of FA1090 Hgb^+ had an unusual amino acid composition, containing 12.2% serine and 11.9% glycine. There was 84.2% similarity and 82.0% identity to the HpuA of DNM2.

One codon after the coding sequence for LAAC was a run of G residues, starting at nucleotide 58 of *hpuA* (Fig. 1). Direct sequencing of *hpuA* PCR products revealed that the FA1090 variant unable to utilize hemoglobin for growth contained only 9 G's in the poly(G) tract, while the hemoglobin-utilizing variant of FA1090 contained 10 G's. Identical results were obtained from each of the four independent PCRs for both the Hgb^- variant and the Hgb^+ variant. PCR products from FA19 Hgb^- and FA19 Hgb^+, two reactions each, also contained 9 G's for the nonhemoglobin-utilizing variant and 10 G's for the hemoglobin-utilizing variant. Since the number of G's in both strains was consistent for the respective variants, the difference in numbers was variant specific and not the result of PCR error. Deletion of 1 G from the 10-G poly(G) tract resulted in a frame shift in the coding sequence for HpuA and led to an immediate stop codon (Fig. 1). Thus, changes in the expression states of *hpuA* involved an altered reading frame register and resulted in switches between the "on phase" and the "off phase" of hemoglobin utilization.

PCR clones and insertional mutants of *hpuA*. Curiously, pUNCH258 and pUNCH259, which were plasmids containing cloned *hpuA* from FA1090 Hgb^- and FA1090 Hgb^+, respectively, both contained 9 G's in the poly(G) tract. It seems that reversion had occurred in the *E. coli* transformation of pUNCH259 and that the selection was skewed to clones containing 9 G's. Insertional mutagenesis of FA1090 Hgb^+ resulted in *hpuB* mutant FA6929 (8) and *hpuA* mutants FA6982 and FA6983. All three mutants contained 10 G's in the poly(G) tract, whereas the *hpuA* insertional mutant of FA1090 Hgb^-, FA6981, contained 9 G's. Thus, gonococcal transformation with a fragment of *hpuA* containing either the Ω interposon or the *aphA*-3 cassette did not alter the poly(G) region. Changes in hemoglobin utilization phenotypes of these transformants reflected changes that resulted from insertional inactivation of the *hpuA* and/or the *hpuB* alleles.

Southern blot analysis of *hpuA* insertional mutants. In order to confirm that each mutant had the appropriate insertion, Southern blotting was performed with three separate DNA probes. One was an 850-bp *hpuB* fragment of the *Eco*RI digest of the plasmid pSM85k (30). The other two were the DNA fragments of *Sma*I-cut Ω interposon (2,082 bp) and *Sma*I-cut *aphA*-3 cassette (787 bp). When chromosomal DNA from either FA1090 Hgb^- or FA1090 Hgb^+ was digested with *Cla*I, the *hpuB* probe hybridized with a DNA fragment of roughly 8 kb. In FA6929, FA6982, or FA6983, the same probe hybridized with fragments of approximately 9 to 10 kb. Due to the large sizes of *Cla*I-digested fragments, exact sizes of labeled fragments were hard to estimate; nevertheless, sizes of fragments from the mutants were appropriately larger than the size of the fragment from the parent (Fig. 2, upper panel). The Ω probe hybridized with FA6982 DNA only (Fig. 2A, lower panel), and the *aphA*-3 probe hybridized with FA6983 DNA only (Fig. 2B, lower panel), at the appropriate size positions as predicted.

Localization of the HpuA protein. Expression of both HpuA and HpuB was iron regulated. While HpuB could be detected in stained gels of whole-cell lysates, total membrane proteins, outer membrane proteins, or hemoglobin-binding proteins (8), HpuA was not readily visible in the Coomassie-stained gels, the silver-stained (51) gels, or autoradiographs of iodinated membrane protein gels (data not shown). However, Western blotting with affinity-purified antibody raised against the C-terminal peptide of HpuA revealed an iron stress-induced 42-kDa band in whole cells (data not shown) and membrane preparations. This band appeared along with the 89-kDa HpuB in iron-stressed FA1090 Hgb^+ (Fig. 3A and B, lanes 2) and FA19 Hgb^+ (data not shown) but was absent in the *hpuA* insertional mutants FA6982 and FA6983 (Fig. 3A and B, lanes 6 and 8). The HpuA of hemoglobin-utilizing variants could be affinity purified from total membrane proteins with immobilized hemoglobin (Fig. 3C, lane 2).

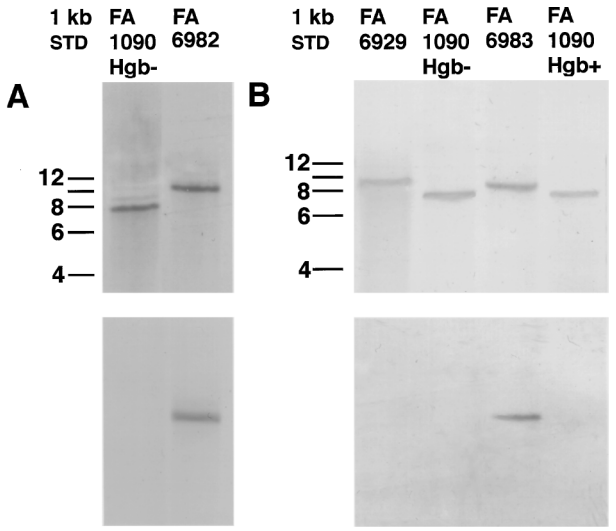

FIG. 2. Southern blot analysis of *hpuA* and *hpuB* insertional mutants. Chromosomal DNAs from FA1090 parent and mutant strains were digested with *Cla*I and transferred bidirectionally to prepare two blots per gel. Hgb^- was the non-hemoglobin-utilizing variant, while Hgb^+ was the hemoglobin-utilizing variant and the recipient for insertional mutation. (A) Blots of DNA fragments from FA6982, a polar insertional *hpuA* mutant, were probed with an *hpuB* fragment (upper panel) and an Ω fragment (lower panel). (B) Blots of DNA fragments from FA6929, an *hpuB* mutant, and FA6983, a nonpolar *hpuA* mutant, were probed with an *hpuB* fragment (upper panel) and an *aphA*-3 fragment (lower panel).

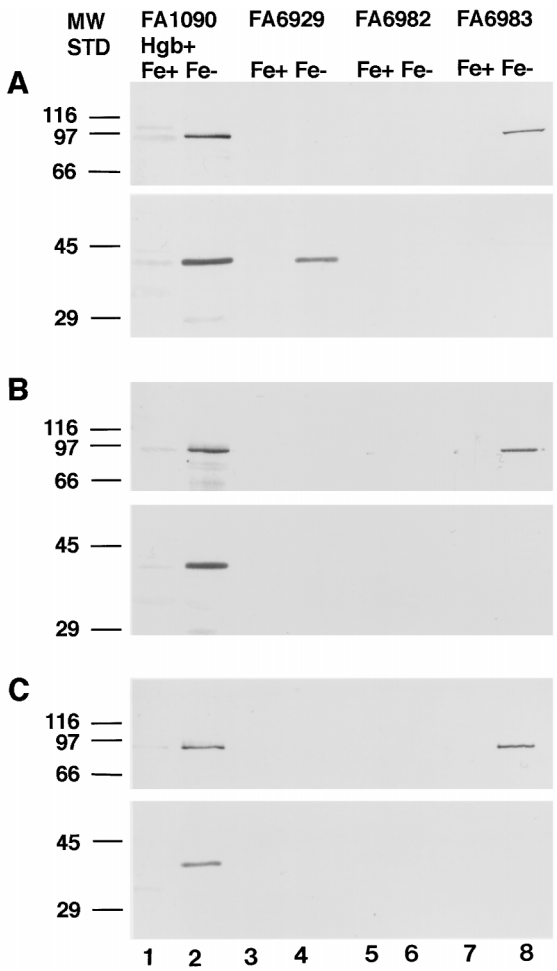

FIG. 3. Detection of HpuA and HpuB among the total membrane proteins (A), the outer membrane proteins (B), and the hemoglobin-binding proteins (C) prepared from the *hpuA* and/or *hpuB* mutants of FA1090 Hgb^+ grown under iron-replete (Fe^+) or iron-stressed (Fe^-) conditions. FA6929 is an *hpuB* insertional mutant, and FA6982 and FA6983 are polar and nonpolar *hpuA* insertional mutants, respectively. Western blots of total membrane proteins, outer membrane proteins, and hemoglobin-binding affinity-purified proteins were cut into two panels each, one probed with affinity-purified anti-HpuB serum (upper panels) and the other probed with affinity-purified anti-HpuA serum (lower panels).

Expression of HpuA and HpuB in mutants. Since we were unable to identify any consensus promoter sequence for *hpuB*, it was expected that insertional mutation of the upstream *hpuA* with the Ω interposon would prevent expression of both HpuA and HpuB. On the other hand, since the *aphA*-3 gene is preceded by translation stop codons in all three reading frames and is followed by a consensus ribosome binding site and a start codon (35), it was expected that successful insertion of the *aphA*-3 cassette in *hpuA* would prevent expression of HpuA but not of HpuB.

HpuA was present in the total membrane proteins prepared from the iron-stressed *hpuB* mutant FA6929 (Fig. 3A, lane 4). However, when HpuB was absent, HpuA could no longer be detected in either the outer membrane protein blot or the hemoglobin-binding protein blot (Fig. 3B and C, lanes 4). The polar insertional *hpuA* mutant, FA6982, showed neither HpuA nor HpuB in all three blots (Fig. 3, lanes 6). The nonpolar insertional *hpuA* mutant, FA6983, did not produce HpuA but did produce HpuB, as expected (Fig. 3A and B, lanes 8). The amount of HpuB produced in the nonpolar *hpuA* mutant might have been reduced, compared to that produced by parent strain, but precise quantification of expression levels was not attempted. The hemoglobin binding ability of HpuB from FA6983 apparently was not affected by the absence of HpuA (Fig. 3C, lane 8).

Hemoglobin binding and utilization of *hpuA* insertional mutants. Iron-stressed, hemoglobin-utilizing FA1090 Hgb^+ bound biotinylated human hemoglobin in a whole-cell dot blot assay (Fig. 4). The binding was inhibited by excess unlabeled hemoglobin but not by excess hemin at a molar ratio of 100:1 (data not shown). The *hpuB* insertional mutant, FA6929, and the polar and nonpolar *hpuA* insertional mutants, FA6982 and FA6983, all demonstrated impaired binding of biotinylated hemoglobin (Fig. 4). None of the *hpuA* or *hpuB* mutants of FA1090 Hgb^+ (FA6929 [$HpuA^+$ $HpuB^-$], FA6982 [$HpuA^-$ $HpuB^-$], or FA6983 [$HpuA^-$ $HpuB^+$]) was able to grow on modified GCB plates using human hemoglobin as the sole iron source (Fig. 5). Nevertheless, all three mutants and both FA1090 Hgb^- and FA1090 Hgb^+ grew well on modified GCB plates containing hemin (data not shown).

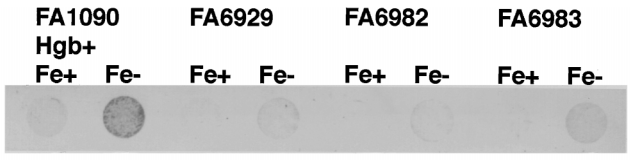

FIG. 4. Dot blot assay showing binding of biotinylated human hemoglobin to iron-replete (Fe^+) and iron-stressed (Fe^-) whole cells. FA1090 Hgb^+ is the parent strain. FA6929 is an *hpuB* insertional mutant, and FA6982 and FA6983 are polar and nonpolar *hpuA* insertional mutants, respectively.

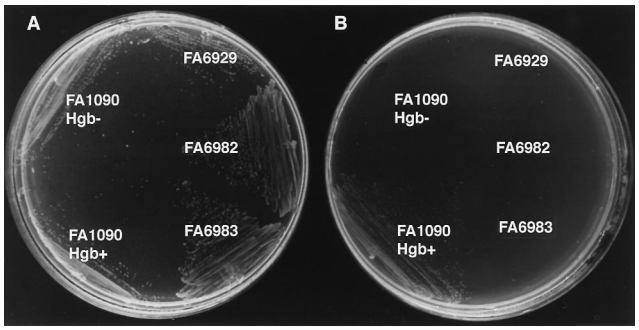

FIG. 5. Growth phenotypes on GCB plate (A) and hemoglobin-Desferal plate (B). FA1090 Hgb^- is the non-hemoglobin-utilizing variant, and FA1090 Hgb^+ is the hemoglobin-utilizing variant. FA6929 is an *hpuB* insertional mutant, and FA6982 and FA6983 are polar and nonpolar *hpuA* insertional mutants of FA1090 Hgb^+, respectively.

DISCUSSION

In order to examine the mechanism underlying the phase variation of hemoglobin utilization by *N. gonorrhoeae*, we studied the expression and function of a 42-kDa outer membrane protein, HpuA, identified in the hemoglobin-utilizing variants of both FA19 and FA1090 grown under iron-stressed conditions. While *hpuA* was located immediately upstream from *hpuB*, the gene encoding the previously reported 89-kDa hemoglobin-binding outer membrane protein (8), we were unable to identify any consensus promoter sequence that might direct the transcription of *hpuB* within the short DNA fragment between the stop codon of *hpuA* and the start codon of *hpuB*. Polar insertional mutation of *hpuA* prevented production of both HpuA and HpuB. Thus, it is most likely that, in *N. gonorrhoeae*, *hpuA* and *hpuB* are comprised in a two-component operon similar to that in *N. meningitidis* (31).

Variation in the expression of gonococcal HpuA and HpuB was associated with variation in the length of a run of G residues at the 5′ end of *hpuA*, located within the sequence encoding the N-terminal amino acids for mature protein. The hemoglobin-utilizing variants contained 10 G's, whereas the non-hemoglobin-utilizing variants contained 9 G's (Fig. 1). When *hpuA* had only 9 G's, a stop codon occurred immediately at the end of the poly(G) tract. In Hgb^+ variants, the 10-G poly(G) tract enabled a change of the translational reading frame and thus the expression of HpuA and HpuB. This apparently explains the high frequency (1×10^{-4} to 2×10^{-3}) of spontaneous Hgb^--to-Hgb^+ variation observed in all tested gonococcal strains (8). Other gonococcal phase-varying systems include Pil (19, 36), PilC (25), Opa (33, 48), and lipooligosaccharide (2). The mechanism for phase variation of HpuA and HpuB is similar to that for Opa, PilC, and lipooligosaccharide in that each apparently uses slipped-strand mispairing to alter the length of a short DNA repeat, which affects translational frame and therefore expression (13, 18, 25, 39, 40).

Although we have been unable to develop a protocol to detect the on-phase-to-off-phase switch of hemoglobin utilization, it is likely that the postulated on-to-off switch also occurs at a relatively high frequency. It is unclear what advantage might be gained by phase variation and iron repression of hemoglobin-binding protein expression. We speculate, however, that phase variation of a hemoglobin-binding protein in gonococci would enable efficient utilization of menstrual hemoglobin. Recently, two genes encoding hemoglobin-binding proteins of *Haemophilus influenzae* (HgpA and HgpB) have been cloned (23, 24, 45). Regions of CCAA nucleotide-repeating units immediately following the putative leader cleavage site were identified in both *hgpA* and *hgpB*. It has been postulated that alteration of the reading frame across the CCAA region of *hgpA* or *hgpB* by strand slippage would lead to production of either protein (23). Thus, the heme-regulated expression of *H. influenzae* hemoglobin-binding proteins might very well be another example of phase variation of hemoglobin-binding proteins.

The gonococcal *hpuA* gene encodes a consensus signal peptidase II cleavage site (21), suggesting that HpuA is lipid modified. The difference between the observed molecular mass (42 kDa) and the calculated molecular mass (36 kDa) is consistent with lipid modification of HpuA. The nonintegral membrane protein characteristics of HpuA were demonstrated in membrane preparations of the $HpuA^+$ $HpuB^-$ mutant FA6929. In the absence of the integral outer membrane protein HpuB, HpuA could not be detected among Sarkosyl-resistant outer membrane proteins (Fig. 3B) or total membrane proteins subjected to hemoglobin-agarose purification (Fig. 3C). On the other hand, the absence of HpuA had no obvious effect on the hemoglobin binding ability of HpuB (Fig. 3C). It is possible that the apparently decreased binding of FA6983 cells to biotinylated hemoglobin (Fig. 4) was due to decreased production of HpuB instead of impaired hemoglobin binding by HpuB (Fig. 3C).

We could not detect hemoglobin binding by HpuA alone in cell-free systems, but HpuA must play a critical role in the utilization of hemoglobin for growth. FA6983, the mutant which expressed HpuB but not HpuA, bound hemoglobin in affinity purification (Fig. 3C) but was unable to grow on media where hemoglobin was the sole source of iron (Fig. 5). Thus, HpuA and HpuB, while showing differences in their abilities to bind hemoglobin, were both required for hemoglobin utilization by gonococci.

Lee (26) reported isolation of two hemin-binding proteins (HmBPs) by affinity purification from gonococci grown under iron-limited conditions. These two HmBPs have observed molecular masses of 44 and 97 kDa, very close to the 42 and 89 kDa of HpuA and HpuB. HmBPs bound to hemin-agarose, and the binding was competitively inhibited by hemoglobin. Growth on hemoglobin was inhibited by a monoclonal antibody directed against the 97-kDa HmBP (26, 29). These results suggest that HmBPs might be identical to HpuA and HpuB. However, our results showed that neither HpuA nor HpuB was required for growth on hemin plates, and in whole-cell dot blot assays, hemoglobin binding by FA1090 Hgb^+ was not affected by excess hemin. These are not the results that would be expected if HpuA and HpuB were identical to the HmBPs. In the current absence of sequence data for the HmBPs, and of specific mutants of the HmBPs, we cannot be certain that HpuA and HpuB are different from the HmBPs.

Gonococci possess multiple specific systems to acquire iron from different sources: transferrin and lactoferrin (4, 5, 11, 34), aerobactin (54), enterobactin (46), hemoglobin (38), and, undoubtedly, heme (14, 15, 27). This presumably reflects the differences in available iron at different body sites and under different physiological conditions (53). Acquisition of iron from transferrin, lactoferrin, and hemoglobin may involve a bipartite receptor system. The operon, *tbpBA*, encoding the outer membrane lipoprotein, TbpB, and the TonB-dependent outer membrane transferrin receptor, TbpA, has been well studied (1, 10, 11). It is also known that there is a gene for a lipoprotein homologous to *tbpB* in meningococci immediately upstream of *lbpA*, which encodes the TonB-dependent lactoferrin receptor LbpA (6, 7, 32, 43). Recently, a similar lipopro-

tein gene has been found in *N. gonorrhoeae*, immediately upstream of *lbpA* (3).

The HpuA-HpuB receptor system for utilization of hemoglobin in *N. gonorrhoeae* is similar in several respects to that of TbpB-TbpA. Gonococcal TbpB, a lipid-modified protein, binds to transferrin in affinity purification but cannot be affinity purified from a TbpA$^-$ mutant in the presence of the detergent Sarkosyl (9). HpuA, the lipoprotein analogous to TbpB, was not Sarkosyl resistant and could be affinity purified only in the presence of HpuB, the integral outer membrane protein analogous to TbpA. This could reflect a physical association between HpuA and HpuB, such as has been proposed for TbpB and TbpA (12). In *N. gonorrhoeae*, a TbpB$^-$ mutant shows reduced binding of transferrin but exhibits growth on transferrin plates; a TbpA$^-$ mutant binds less transferrin and does not grow on transferrin plates (1). In these respects, the gonococcal TbpB-TbpA system does not correspond to what we have described for HpuA and HpuB; however, in *N. meningitidis*, both TbpB$^-$ and TbpA$^-$ mutants are unable to grow on transferrin-bound iron (22), analogous to the results for the gonococcal HpuA-HpuB system.

The importance of both HpuA and HpuB in gonococcal hemoglobin utilization has been demonstrated, but many questions remain to be answered concerning the exact function and location of HpuA and HpuB. A better understanding of how they interact to facilitate iron uptake from hemoglobin will be the aim of future work.

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REFERENCES

1. **Anderson, J. E., P. F. Sparling, and C. N. Cornelissen.** 1994. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. J. Bacteriol. **176:**3162–3170.
2. **Apicella, M. A., M. Shero, G. A. Jarvis, J. M. Griffiss, R. E. Mandrell, and H. Schneider.** 1987. Phenotypic variation in epitope expression of the *Neisseria gonorrhoeae* lipooligosaccharide. Infect. Immun. **55:**1755–1761.
3. **Biswas, G., and P. F. Sparling.** 1997. Personal communication.
4. **Biswas, G. D., and P. F. Sparling.** 1995. Characterization of *lbpA*, the structural gene for a lactoferrin receptor in *Neisseria gonorrhoeae*. Infect. Immun. **63:**2958–2967.
5. **Blanton, K. J., G. D. Biswas, J. Tsai, J. Adams, D. W. Dyer, S. M. Davis, G. G. Koch, P. K. Sen, and P. F. Sparling.** 1990. Genetic evidence that *Neisseria gonorrhoeae* produces specific receptors for transferrin and lactoferrin. J. Bacteriol. **172:**5225–5235.
6. **Bonnah, R. A., H. Wong, and A. B. Schryvers.** 1996. Genetic and serological analysis of lactoferrin receptors in the Neisseria: evidence for the antigenically conserved nature of LbpB, abstr. p. 206, p. 560. *In* W. Zollinger, C. Frasch, and C. Deal (ed.), Abstracts of the Tenth International Pathogenic *Neisseria* Conference.
7. **Bonnah, R. A., R.-H. Yu, and A. B. Schryvers.** 1995. Biochemical analysis of lactoferrin receptors in the *Neisseriaceae*: identification of a second bacterial lactoferrin receptor. Microb. Pathog. **19:**285–297.
8. **Chen, C.-J., P. F. Sparling, L. A. Lewis, D. W. Dyer, and C. Elkins.** 1996. Identification and purification of a hemoglobin-binding outer membrane protein from *Neisseria gonorrhoeae*. Infect. Immun. **64:**5008–5014.
9. **Cornelissen, C., and P. F. Sparling.** 1996. Binding and surface exposure characteristics of the gonococcal transferrin receptor are dependent on both transferrin-binding proteins. J. Bacteriol. **178:**1437–1444.
10. **Cornelissen, C. N., G. D. Biswas, J. Tsai, D. K. Paruchuri, S. A. Thompson, and P. F. Sparling.** 1992. Gonococcal transferrin-binding protein 1 is required for transferrin utilization and is homologous to TonB-dependent outer membrane receptors. J. Bacteriol. **174:**5788–5797.
11. **Cornelissen, C. N., and P. F. Sparling.** 1994. Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. Mol. Microbiol. **14:**843–850.
12. **Cornelissen, C. N., J. E. Anderson, and P. F. Sparling.** 1997. Energy-dependent changes in the gonococcal transferrin receptor. Mol. Microbiol. **26:**25–35.
13. **Danaher, R. J., J. C. Levin, D. Arking, C. L. Burch, and R. Sandlin.** 1995. Genetic basis of *Neisseria gonorrhoeae* lipooligosaccharide antigenic variation. J. Bacteriol. **177:**7275–7279.
14. **Desai, P. J., R. Nzeribe, and C. A. Genco.** 1995. Binding and accumulation of hemin in *Neisseria gonorrhoeae*. Infect. Immun. **63:**4634–4641.
15. **Dyer, D. W., E. P. West, and P. F. Sparling.** 1987. Effects of serum carrier proteins on the growth of pathogenic *Neisseriae* with heme-bound iron. Infect. Immun. **55:**2171–2175.
16. **Elkins, C.** 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. Infect. Immun. **63:**1241–1245.
17. **Elkins, C., N. H. Carbonetti, V. A. Varela, D. Stirewalt, D. G. Klapper, and P. F. Sparling.** 1992. Antibodies to N-terminal peptides of gonococcal porin are bactericidal when gonococcal lipopolysaccharide is not sialylated. Mol. Microbiol. **6:**2617–2628.
18. **Gotschlich, E. C.** 1994. Genetic locus for the biosynthesis of the variable portion of *Neisseria gonorrhoeae* lipooligosaccharide. J. Exp. Med. **180:**2181–2190.
19. **Hagblom, P., E. Segal, E. Billyard, and M. So.** 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature **315:**156–158.
20. **Harlow, E., and D. Lane.** 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Hayashi, S., and H. C. Wu.** 1990. Lipoprotein in Bacteria. J. Bioenerg. Biomembr. **22:**451–471.
22. **Irwin, S. W., C. Y. Averil, C. Y. Cheng, and A. B. Schryvers.** 1993. Preparation and analysis of isogenic mutants in the transferrin receptor protein genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. Mol. Microbiol. **8:**1125–1133.
23. **Jin, H., Z. Ren, J. M. Pozsgay, C. Elkins, P. W. Whitby, D. J. Morton, and T. L. Stull.** 1996. Cloning of a DNA fragment encoding a heme-repressible hemoglobin-binding protein outer membrane protein from *Haemophilus influenzae*. Infect. Immun. **64:**3134–3141.
24. **Jin, H., Z. Ren, P. W. Whitby, D. J. Morton, and T. L. Stull.** 1997. Genetic analysis of a hemoglobin binding protein (HgpA) of *Haemophilus influenzae*, abstr. B-223, p. 67. *In* Abstracts of the 97th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
25. **Jonsson, A. B., G. Nyberg, and S. Normark.** 1991. Phase variation of gonococcal pili by frameshift mutation in pilC, a novel gene for pilus assembly. EMBO J. **10:**477–488.
26. **Lee, B. C.** 1992. Isolation of haemin-binding proteins of *Neisseria gonorrhoeae*. J. Med. Microbiol. **36:**121–127.
27. **Lee, B. C.** 1995. Quelling the red menace: haem capture by bacteria. Mol. Microbiol. **18:**383–390.
28. **Lee, B. C., and P. Hill.** 1992. Identification of an outer-membrane haemoglobin-binding protein in *Neisseria meningitidis*. J. Gen. Microbiol. **138:**2647–2656.
29. **Lee, B. C., and S. Levesque.** 1997. A monoclonal antibody directed against the 97-kilodalton gonococcal hemin-binding protein inhibits hemin utilization by *Neisseria gonorrhoeae*. Infect. Immun. **65:**2970–2974.
30. **Lewis, L. A., and D. W. Dyer.** 1995. Identification of an iron-regulated outer membrane protein of *Neisseria gonorrhoeae* involved in the utilization of hemoglobin complexed to haptoglobin. J. Bacteriol. **177:**1299–1306.
31. **Lewis, L. A., E. Gray, Y.-P. Wang, B. A. Roe, and D. W. Dyer.** 1997. Molecular characterization of *hpuAB*, the haemoglobin-haptoglobin-utilization operon of *Neisseria meningitidis*. Mol. Microbiol. **23:**737–749.
32. **Lewis, L. A., K. H. Rohde, B. Behrens, E. Gray, S. I. Toth, B. A. Roe, and D. W. Dyer.** 1996. Molecular analysis of *lbpAB* encoding the two-component meningococcal lactoferrin receptor, abstr. P-214, p. 557. *In* W. Zollinger, C. Frasch, and C. Deal (ed.), Abstracts of the Tenth International Pathogenic *Neisseria* Conference.
33. **Mayer, L. W.** 1982. Rates of in vitro changes in gonococcal colony opacity phenotypes. Infect. Immun. **37:**481–485.
34. **McKenna, W. R., P. A. Mickelsen, P. F. Sparling, and D. W. Dyer.** 1988. Iron uptake from lactoferrin and transferrin by *Neisseria gonorrhoeae*. Infect. Immun. **56:**785–791.
35. **Ménard, R., P. J. Sansonetti, and C. Parsot.** 1993. Nonpolar mutagenesis of the *ipa* genes defines IpaB, IpaC, and IpaD as effectors of *Shigella flexneri* entry into epithelial cells. J. Bacteriol. **175:**5899–5906.
36. **Meyer, T. F., N. Mlawer, and M. So.** 1982. Pilus expression in *Neisseria gonorrhoeae* involves chromosomal rearrangement. Cell **30:**45–52.
37. **Mickelsen, P. A., E. Blackman, and P. F. Sparling.** 1982. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from lactoferrin. Infect. Immun. **35:**915–920.
38. **Mickelsen, P. A., and P. F. Sparling.** 1981. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. Infect. Immun. **33:**555–564.

39. **Muralidharan, K., A. Stern, and T. F. Meyer.** 1987. The control mechanism of opacity protein expression in the pathogenic Neisseriae. Antonie Leeuwenhoek **53:**435–440.
40. **Murphy, G. L., T. D. Connell, D. S. Barritt, M. Koomey, and J. G. Cannon.** 1989. Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. Cell **56:**539–547.
41. **Nachamkin, I., J. G. Cannon, and R. S. Mittler.** 1981. Monoclonal antibodies against *Neisseria gonorrhoeae*: production of antibodies directed against a strain-specific cell surface antigen. Infect. Immun. **33:**555–564.
42. **Parekh, B. S., P. W. Schwimmbeck, and M. J. Buchmeier.** 1989. High efficiency immunoaffinity purification of antipeptide antibodies on thiopropyl Sepharose immunoadsorbents. Peptide Res. **2:**249–253.
43. **Petterson, A., T. Prinz, P. van der Ley, J. T. Poolman, and J. Tommassen.** 1996. Characterization of the meningococcal lactoferrin receptor, abstr. P-221, p. 587. *In* W. Zollinger, C. Frasch, and C. Deal (ed.), Abstracts of the Tenth International Pathogenic *Neisseria* Conference.
44. **Prentki, P., and H. M. Krisch.** 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene **29:**303–313.
45. **Ren, Z., H. Jin, D. J. Morton, and T. L. Stull.** 1997. Cloning of *hgpB*, a gene encoding a second hemoglobin binding protein of *Haemophilus influenzae*, abstr. B-227, p. 67. *In* Abstracts of the 97th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
46. **Rutz, J. M., T. Abdullah, S. P. Singh, V. I. Kalve, and P. E. Klebba.** 1991. Evolution of the ferric enterobactin receptor in gram-negative bacteria. J. Bacteriol. **173:**5964–5974.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
48. **Stern, A., M. Brown, P. Nickel, and T. F. Meyer.** 1986. Opacity genes in *Neisseria gonorrhoeae*: control of phase and antigenic variation. Cell **47:**61–71.
49. **Stojiljkovic, I., V. Hwa, S. L. de Martin, P. O'Gaora, X. Nassif, F. Heffon, and M. So.** 1995. The *Neisseria meningitidis* hemoglobin receptor: its role in iron utilization and virulence. Mol. Microbiol. **15:**531–541.
50. **Stojiljkovic, I., J. Larson, V. Hwa, S. Anic, and M. So.** 1996. HmbR outer membrane receptors of pathogenic *Neisseria* spp.: iron-regulated, hemoglobin-binding proteins with a high level of primary structure conservation. J. Bacteriol. **178:**4670–4678.
51. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
52. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42:**45–66.
53. **West, S. E. H., and P. F. Sparling.** 1985. Response of *Neisseria gonorrhoeae* to iron limitation: alterations in expression of membrane proteins without apparent siderophore production. Infect. Immun. **47:**388–394.
54. **West, S. E. H., and P. F. Sparling.** 1987. Aerobactin utilization by *Neisseria gonorrhoeae* and cloning of a genomic DNA fragment that complements *Escherichia coli fhuB* mutations. J. Bacteriol. **169:**3414–3421.

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